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Effect of Laser Irradiation on the Growth and Development of Fetal Mouse Limbs in an In Vitro Model

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Background and Objective: **The purpose of the present study was to examine the effects of laser irradiation on the growth and development of fetal limb tissue.**
Study Design/Materials and Methods: **Day 14 fetal mouse limbs (n = 168) were irradiated with gallium arsenide laser (904 nm, spot size = 0.002 cm^2, pulse duration = 200 nanoseconds, peak power = 30 mW) for 1 minute each day while being maintained in an organ culture system for 3 or 5 days at the following energy densities [0 (control), 0.23, 1.37, 2.75, 3.66, and 4.58 J/cm^2].**
Results: **Computer image analysis of photographic images showed that there was a significant inhibition ($P < 0.05$) of new tissue growth after administration of lower energy densities of laser (0.23 and 1.37 J/cm^2). These low-energy densities of laser irradiation also produced increased dermal cell number and collagen fiber thickness as assessed with qualitative histologic analysis of limb development by a blinded observer. Quantitative analysis of collagen distribution by color densitometric analysis of tissue sections stained with sirus red and fast green confirmed that there was a significantly greater ($P < 0.05$) amount of collagen present in the dermis of limbs treated with low-energy densities of laser (0.23 and 1.37 J/cm^2).**
Conclusions: **Laser irradiation directly affected the growth and development of day 14 fetal mouse limbs in an organ culture system. Lasers Surg. Med. 24:285–295, 1999.**

Key words: collagen; computer image analysis; histologic characteristics; laser safety; pregnancy

INTRODUCTION

A-low energy laser is one that produces an insignificant temperature elevation of less than 0.1–0.5°C in tissue [1]. Low-energy lasers commonly used in physiotherapy for wound healing and pain modulation include the gallium-arsenide (GaAs) and the helium-neon (HeNe) lasers. Randomized controlled trials indicate that laser is an effective modality for the treatment of musculoskeletal conditions including osteoarthritis, rheumatoid arthritis, lateral epicondylitis, rotator cuff tendinitis, tempromandibular pain dysfunction, and carpal tunnel syndrome [2–8]. Although the mechanism and biological basis for the pain-attenuating effects of laser irradiation is poorly understood, well-controlled human studies have suggested that laser irradiation can alter nerve conduction, skin resistance over trigger points, and pain threshold [9–11].

Decreasing connective tissue strength and rigidity, resulting in ligament and joint laxity

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during pregnancy, have been implicated as contributing factors in the etiology of a plethora of musculoskeletal complications. Despite the painful and disabling nature of these conditions, the use of therapeutic modalities has been discouraged because of the unknown risks to the developing fetus or the pregnant mother. Therefore, treatment of obstetric patients has been limited to patient education and preventive measures in the hope that symptoms would resolve with the completion of pregnancy. Laser has been used by physical therapists to treat successfully a variety of painful musculoskeletal conditions [2–8] and may be a viable adjunct to current strategies used in the management of pregnancy-induced musculoskeletal conditions. However, the use of laser for analgesia and tissue healing during pregnancy is currently contraindicated because of the potential risks posed to the developing fetus in utero. No studies to date have examined the effects of laser irradiation on fetal growth and development. The purpose of the present study was to examine the effects of laser irradiation on the growth and development of fetal limb tissue as an initial attempt to support or refute the existing contraindication.

Fetal mouse limb development occurs between gestation days 12 and 20 (term = 21 days) in a proximodistal direction, with the hindlimb lagging behind the forelimb in development by half a day [12]. By gestation day 14, individual fingers have separated in the forelimb and deep indentations are present between the developing toes in the hindlimb. Numerous hair follicles are also present in the skin at this time [13]. By gestation day 15, the long bones of the extremities show only periosteal ossification [13]. Epidermal and dermal development occur later in gestation, and by day 20 the epidermis consists of four to five cell layers, with the first signs of a distinct dermis becoming apparent [12]. This pattern of mouse limb development is similar to that which occurs in human fetuses. Because fetal mouse limb development occurs much more rapidly, it is an ideal model for examining the effects of laser irradiation on limb growth and development.

MATERIALS AND METHODS

Animals

In this study, an inbreed strain (CD-1) of pregnant mice (n = 6) was used 14 days after breeding (Charles River Laboratories, St. Constant, QC, Canada). Upon arrival, the animals were housed in cages with controlled lighting and temperature. Food and water were provided ad libitum, and 72 hours were allowed for the animals to recover from traveling prior to experimental intervention. At day 14 of gestation, the pregnant mice were killed individually with an overdose of carbon dioxide gas in an induction chamber. Upon cessation of respiratory effort, the mouse was removed from the chamber and the abdomen was prepared with 70% alcohol solution. Laparotomy and hysterectomy were performed under aseptic conditions to remove uterine contents, which were placed in ice-cooled Hank's Balanced Salt Solution. Using a WILD Lietz stereomicroscope (Leica, Germany) at 16× magnification, the uterine and fetal membrane tissue were resected, followed by amputation of forelimbs (n = 84) and hindlimbs (n = 84) close to the body wall. Ipsilateral forelimbs and hindlimbs were then placed into single-organ culture systems that had been previously prepared and heated to incubator temperature (37°C, 95% humidity, 5% CO_2/95% air mixture). All experimental procedures and methods of animal handling were approved by the Institutional Animal Care Committee and complied with Canadian Council on Animal Care guidelines.

Organ Culture System

Day 14 limbs were maintained for three or five days while receiving daily 1-minute laser irradiation in a previously validated organ culture system, which has been described elsewhere [14,15]. Briefly, the system consisted of an outer standard Petri dish and an inner organ culture dish (Falcon, Lincoln Park, NJ) filled with sterile distilled water for maintenance of limb humidity. The inner well of the organ culture dish supported a sterile stainless-steel wire-mesh grid (Industrial Wire Products, Los Angeles, CA) on which limbs were partly submerged in serum-free BGJb media (Fitton-Jackson Modification, Life Technologies, Bethesda, MD) supplemented with full-spectrum antibiotics (penicillin G 100 U/ml, streptomycin 0.1 mg/ml, amphotericin B 0.25 mg/ml; Sigma Chemical Co., St. Louis, MO) and ascorbic acid (1 μg/ml). Medium was substituted on alternate days. Organ culture dishes were stored in an incubator set at 37°C, 95% humidity, and 5% carbon dioxide and 95% air mixture.

Laser Treatment

Organ culture dishes containing ipsilateral forelimbs and hindlimbs were randomly placed

TABLE 1. Laser Treatment Groups

Energy density (J/cm^2)[a]	Frequency (Hz)	Average power output (mW)
0 (control)	0	0
0.23	500	3
1.37	3,000	18
2.75	6,000	36
2.75	8,000	36
3.66	8,000	48
4.58	10,000	60

[a]The energy density (J/cm^2) represents the therapeutic dose incident on the target tissue. Calculation of energy density assumes an effective radiating area of 1 cm^2.

into one of seven laser treatment groups (Table 1). All limbs maintained in an organ culture system were exposed to laser irradiation for 1 minute each day for up to three or five days. The limbs were randomly assigned to receive energy densities of 0 (control), 0.23, 1.37, 2.75, 3.66, or 4.58 J/cm^2 through an apparatus that was assembled in a tissue culture hood at room temperature (Table 1). A GaAs laser was used (904 nm, spot size = 0.002 cm^2, pulse duration = 200 nanoseconds, peak power = 30 mW; IMLS MD5 Therapeutic Laser Systems, Theralase Inc., Mississauga, ON, Canada). The tip of the laser probe, which contained the semiconductor diode, was lowered into direct contact with the cover of the organ culture dish to ensure that the laser beam was consistently delivered at a 90° angle and from a distance of 0.5 cm to the dorsal surface of the limb. The laser was calibrated prior to beginning the experiment, and a beam test was performed prior to daily treatment. Calculation of energy density assumed an effective irradiating area of 1 cm^2.

Tissue Processing and Staining

Limbs were randomly selected to be removed from culture after either three or five days of laser irradiation. At the time of removal, limbs were fixed in 10% buffered formalin and assigned a numerical code to conceal identification of the laser treatment group. After fixation, the limbs were embedded in paraffin and cut on a microtome to 5 μm thickness and placed onto slides. After performing standard histologic processing techniques, as described elsewhere [16,17], cross sections were stained with the classic hematoxylin and eosin (H&E) technique and a picro sirus-red fast-green (SRFG) technique. Hematoxylin binds to nuclei and eosin to cytoplasmic components. Sirus red binds to collagen and fast green to noncollagenous protein [18]. This SRFG staining technique, described in detail elsewhere, has been used to accurately quantify the amount of dermal collagen present in fetal limbs [14,19].

Data Collection

This was a multilevel, completely randomized study that compared six laser-treated groups with a control (sham) laser group. An observer who was blinded to the laser treatment groups collected all data. The dependent variables included limb morphometry (change in limb length and amount of new tissue growth), qualitative histologic observations of the epidermis, dermis, and bone, and quantitative determination of epidermal thickness and dermal and bone collagen/noncollagenous protein ratios.

Limb morphometry. Photographic images of limbs on the first, third, and fifth days in culture after laser irradiation were captured with a 930 DXC RGB Sony video camera and a WILD Lietz stereomicroscope at 10× magnification. All images were assessed quantitatively with computerized image analysis (Northern Exposure, Empix Imaging Inc., Mississauga, ON, Canada) with the gray-level thresholding technique. Limb morphometry was quantified by measuring the length and the amount of new tissue growth of each limb (Fig. 1). The length of each limb was defined as the maximum distance between the tip of the third digit and the amputated end of the limb and was determined by using the distance measuring tool of the image analysis program (Northern Exposure), previously calibrated to 1 mm and having a resolution of 1 μ. New tissue growth was defined as tissue not observed on day 1 but discernible at the amputated end of a limb on day 3 or 5. The new tissue growth present was traced by using the tracing selection tool to provide an estimate of the amount of new tissue growth. Each limb was measured twice for each dependent variable, and an average was calculated and used for statistical analysis. Differences in initial limb length due to variation in the amputation level were accounted for by measuring the change in the total length of the limb that occurred between days 1 and 3 and between days 1 and 5. Data are expressed as the mean ± SEM of the change in the length and the amount of new tissue growth of limbs from days 1 to 3 and from days 1 to 5.

Histologic analysis. Only cross sections containing the radius and ulna or tibia and fibula were assessed, thereby permitting evaluation of

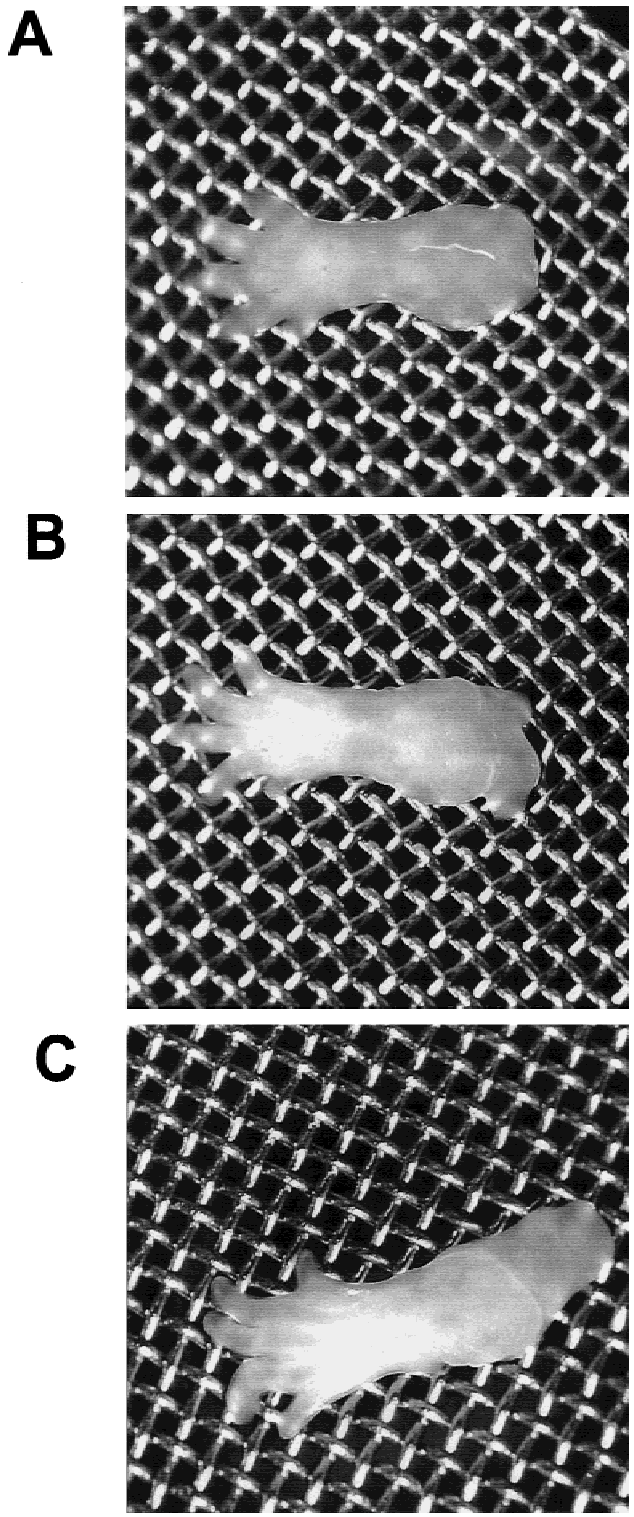

Fig. 1. Fetal mouse limbs (gestation day 14) shown on days 1 (**A**), 3 (**B**), and 5 (**C**) after laser irradiation in organ culture. Stereomicroscope, 10× magnification.

tissue samples derived from a similar region of each limb. Evaluations were completed under a consistent light intensity by an experienced observer who was blinded to the laser treatment groups. All measurements were made within a predetermined area on the dorsal surface of the limb between the lateral and lower most borders of both bones of each cross section.

Qualitative histologic analysis. Qualitative histologic analysis of the stage of limb development was performed with an ordinal scale developed for the present study. All cross sections were evaluated according to predetermined criteria based on changes in limb development that occur normally in vivo. The epidermal, dermal, and bone layers were evaluated under 40× magnification and assigned numerical scores on each cross section according to predetermined criteria (Table 2). Cross sections were evaluated on two separate occasions to provide an assessment of intrascorer reliability.

The thickness of the epidermis, the presence of hair follicles, and the shapes of epidermal cells observed were evaluated in cross sections stained with H&E and SRFG. The presence of round epidermal cells and an increase in the epidermal thickness and in the number of hair follicles were considered to be indicators of limb development and assigned a score of 3. The dermis was evaluated for the quantity and shape of dermal cells in cross sections stained with H&E. The amount and thickness of collagen fibers and the distribution of collagen were evaluated in cross sections stained with SRFG. An increase in the number of large round dermal cells and increases in the number and thickness of collagen fibers were considered to be indicators of greater limb development and assigned a score of 3.

Bone tissue was evaluated for bone maturity and canal number by using limb sections stained with both H&E and SRFG. The number of cells and the distribution of collagen were evaluated by using sections stained with H&E and SRFG, respectively. A decrease in the number of cells and canals and the presence of concentrated areas of intensely stained collagen localized to the bone periphery were considered as indicators of greater limb development and assigned a score of 3. Data are expressed as the mean ± SEM for each indicator of the stage of limb development and for each tissue type (epidermis, dermis, and bone) after 3 and 5 days of laser irradiation.

Quantitative histologic analysis: Epidermal thickness. The thickness of the epidermis was de-

TABLE 2. Criteria for Qualitative Histologic Analysis of the Stage of Fetal Limb Development

	Predetermined criteria and assigned scores		
Indicators of limb development	1	2	3
Epidermis			
Thickness	Thin	Normal	Thick
Cells	Squamous	Combination	Round
Hair follicles	Absent		Present
Dermis			
Fiber amount	Minimal	Moderate	Predominant
Fiber thickness	Fine	Combination	Coarse
Cell amount	Minimal	Moderate	Predominant
Cell shape	Sigmoid/flat	Combination	Large/round
Bone			
Maturity	Messenchymal condensation	Cartilage	Bone
Cell amount	Predominant	Moderate	Minimal
Canal number	Absent	Predominant	Minimal
Collagen distribution	Diffuse pink haze		Dense red periphery

termined for selected cross sections from each treatment group stained with either H&E or SRFG by using computer image analysis (Northern Exposure) with the distance-measuring tool under 20× magnification. This tool provided an estimate of the width of the epidermal layer, referred to as the thickness of the epidermis. An average of three measurements recorded within a predetermined area on the dorsal surface of each limb between the radius and ulna or between the tibia and fibula was calculated and used for statistical analysis. Data are expressed as the mean ± SEM of the epidermal thickness of limbs on days 3 and 5 in culture after laser irradiation.

Quantitative histologic analysis: Collagen/noncollagenous protein ratios. Collagen/noncollagenous protein ratios were determined in the dermis and in the bones of selected cross sections from each treatment group stained with SRFG. Computerized image analysis was used to obtain a color densitometric measurement of the amount of red and green stains, representing collagen and non-collagenous protein, respectively. This ratio of the color density of the red collagen stain over the color density of the green protein stain was expressed as a collagen/noncollagenous protein ratio. Three measurements were recorded in the dermis, and an average was calculated and used for statistical analysis. Two measurements were recorded from a randomly selected region of both long bones present in each cross section analyzed. The bone demonstrating the highest average value for the collagen/noncollagenous protein ratio in a given tissue section was retained for statistical analysis. Data are expressed as the mean ± SEM of the collagen/noncollagenous protein ratios in the dermis and in the bones of limbs on days 3 and 5 in culture after laser irradiation.

Statistical Analysis

The effect of laser irradiation on the growth and development of fetal limb tissue was assessed by testing for significant differences ($P < 0.05$) between dependent variables obtained from all laser-irradiated groups and those obtained from limbs in the control group. Significant differences in dependent variables were accepted as an indication of the effect of laser irradiation on the growth and development of fetal limb tissue. One-way analysis of variance followed by the Tukey HSD method of multiple comparisons was used to compare group means of limbs irradiated with laser and limbs that received sham laser. A 0.05 significance level was used for the analysis of variance. A *t*-test was used to identify significant differences ($P < 0.05$) in the dependent variables of limbs treated for three days vs. limbs treated for five days for each laser treatment group. Data derived from limbs in groups irradiated at energy densities of 0.23 and 1.37 J/cm^2 were not distributed normally. In these two cases, a Mann-Whitney rank sum test was used to identify significant differences ($P < 0.05$) in the dependent variables of limbs irradiated for three days vs. limbs irradiated for five days. Qualitative histologic data were analyzed by using the Kruskal-Wallis one-way analysis of variance on ranks, followed by the Dunn method of multiple comparisons to compare the average histologic scores obtained from limbs irradiated with laser with limbs that received sham control laser. A 0.05 significance level was used for the analysis of vari-

ance. The Kruskal-Wallis one-way analysis of variance on ranks is a distribution free statistic based on ranks that controls for the lack of a normal distribution of the data [20]. Intrascorer reliability of cross sections undergoing qualitative histologic analysis was assessed with the ICC equation (1,1). The type of ICC selected was based on a one-way analysis of variance as described by Shrout and Fleiss [21]. All analyses were performed with Jandel Sigma Stat (version 2.0) statistical software.

RESULTS

Limb Morphometry

Analysis of the change in the length of limbs showed that all limbs treated for 3 days increased in length compared with limb length on day 1. Limb length continued to increase from 18.2 μ ± 5.0 on day 3 to 23.2 μ ± 8.6 on day 5 in limbs receiving sham laser irradiation. The amount of increase in the length of limbs that occurred between 3 and 5 days in limbs receiving sham laser was not statistically different when compared with that which occurred in those limbs irradiated with laser at any of the energy densities used in this study. Although the length of limbs increased in all the treatment groups between three and five days of culture, this increase was statistically significant in only the 2.75 J/cm^2 group (Fig. 2A). On day 3 of laser irradiation, administration of lower energy densities of laser (0.23 and 1.37 J/cm^2) produced a significant ($P < 0.05$) inhibitory effect on the amount of new tissue growth present in limbs compared with sham treated limbs (Fig. 2B). Limbs irradiated with the highest energy density of laser (4.58 J/cm^2) also had significantly ($P < 0.05$) less new tissue growth compared with sham irradiated limbs on day 3. There was a further increase in the amount of new tissue growth of limbs in all groups by day 5, with the exception of limbs irradiated at 3.66 J/cm^2. However, the amount of new tissue growth was not significantly different between sham control and laser irradiated groups at day 5.

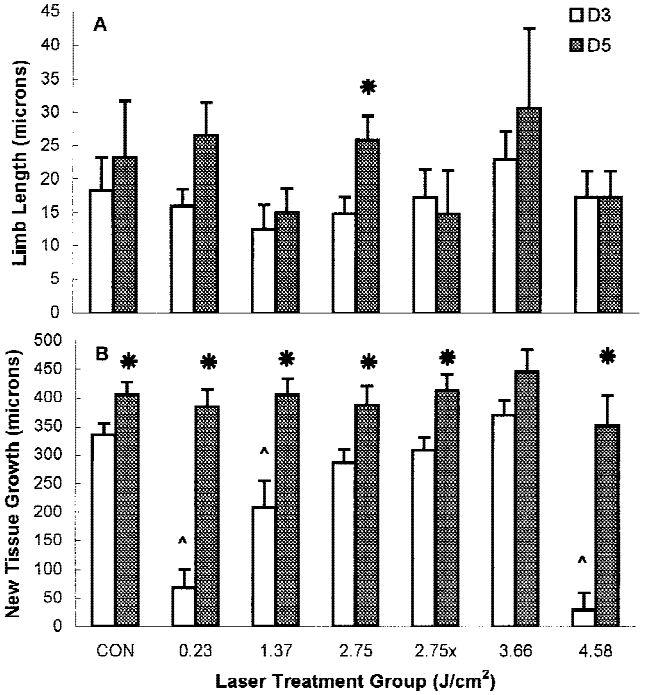

Fig. 2. Effect of laser irradiation on limb morphometry. Effects of laser irradiation on the change in the length (**A**) and the amount (**B**) of new tissue growth of fetal limbs after three (D3; open bars) and five (D5; solid bars) days in organ culture. Changes in limb length and new tissue growth were determined by subtracting estimates of limb length and new tissue growth on day 3 or 5 from those on day 1. Bars represent the mean ± SEM of the change in limb length (A) and amount of tissue growth (B). Carats indicate statistically significant ($P < 0.05$) differences between sham controls and laser-irradiated groups. Asterisks indicate statistically significant ($P < 0.05$) differences between three and five days of culture.

Histologic Analysis

Qualitative histologic analysis. A high degree of intrascorer reliability was demonstrated for repeated evaluations of tissue sections (ICC = 0.98). Histologic scores obtained from the epidermis and bone of limbs did not show significant differences between sham control and laser-irradiated groups at either day 3 or 5 in culture (Fig. 3A,D). Analysis of the histologic scores assigned to the dermis showed a significant effect of laser irradiation on two of the indicators of limb development: (a) the amount of dermal cells and (b) the thickness and amount of dermal collagen fibers. The amount of cells present in the dermis on day 3 of culture was significantly ($P < 0.05$) increased in limbs irradiated with lower energy densities of laser (0.23, 1.37, and 2.75 J/cm^2) compared with sham controls (Fig. 3B). Also on day 3, significant ($P < 0.05$) increases in the thickness and amount of collagen fibers was found in the dermis of limbs irradiated with lower energy densities of laser (0.23 and 1.37 J/cm^2) compared with the sham controls (Fig. 3C). Despite these dramatic histologic changes observed in the dermis of limbs irradiated with lower levels of laser for three days, there was no significant difference in the histologic scores assigned to laser irradiated

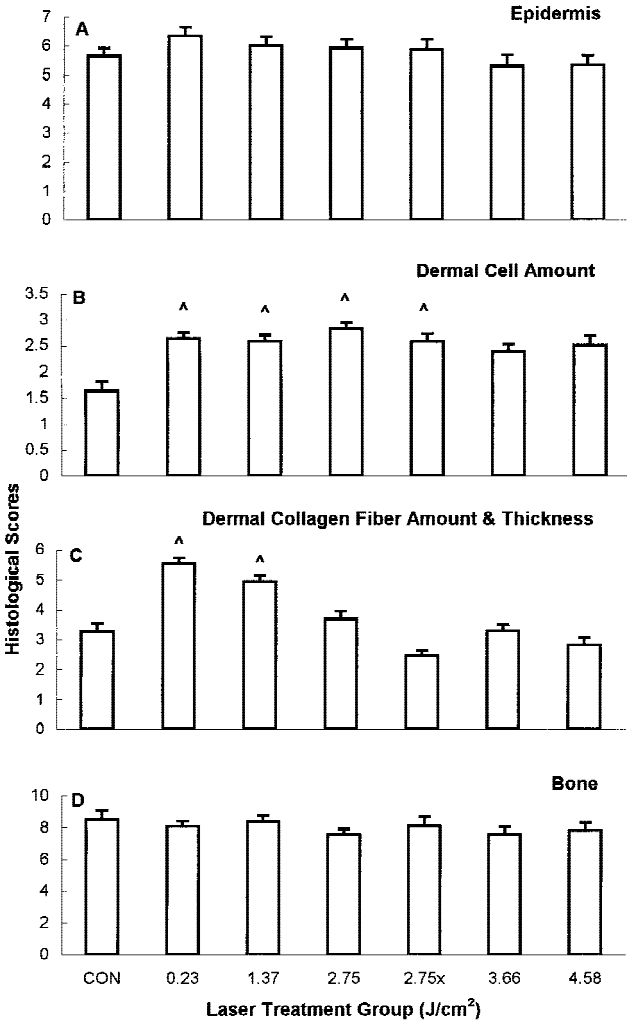

Fig. 3. Effect of laser irradiation on the growth and development of fetal tissue. Effect of laser irradiation on the epidermis (**A**), dermal cell count (**B**), dermal collagen fiber thickness and amount (**C**), and bone development of fetal limbs (**D**) after 3 days in culture. Bars represent the mean ± SEM of the histologic scores assigned to limbs based on predetermined criteria (Table 2). Carats indicate statistically significant ($P < 0.05$) increases in laser-irradiated limbs at increasing energy densities (0.23, 1.37, 2.75, 2.75×, 3.66, and 4.58 J/cm^2) vs. sham controls (CON).

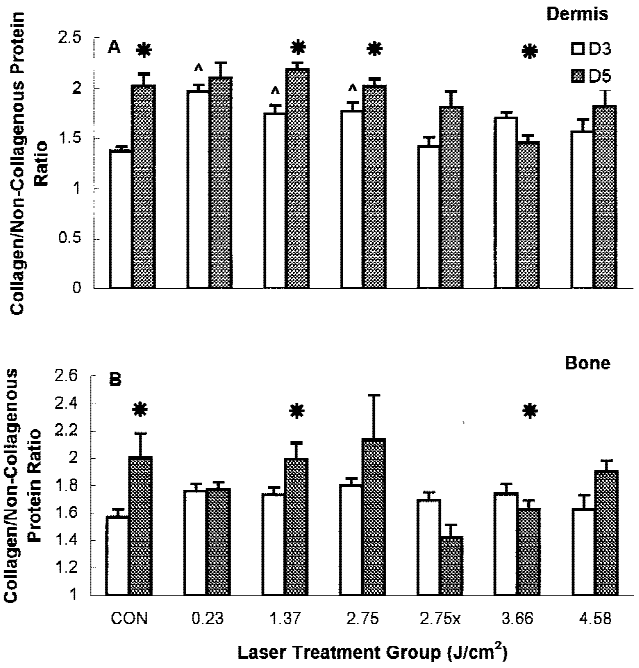

Fig. 4. Effect of laser irradiation on collagen distribution. Effect of laser irradiation on the collagen/noncollagenase protein ratios in the dermis (**A**) and in the bones (**B**) of fetal limbs after three (D3; open bars) and five (D5; solid bars) days in organ culture. Bars represent the means ± SEM of the collagen/noncollagenase protein ratio as determined by color densitometric measurements with computer image analysis. Carats indicate statistically significant ($P < 0.05$) increases in the collagen/noncollagenase protein ratio of limbs irradiated with increasing energy densities of laser (0.23, 1.37, 2.75, 2.75×, 3.66, and 4.58 J/cm^2) vs. sham controls (CON). Asterisks indicate statistically significant ($P < 0.05$) increases in the dermal collagen/noncollagenase protein ratios between limbs irradiated for three days vs. those irradiated at the same energy densities for five days for each laser group.

limbs by five days in culture compared with sham controls receiving similar treatments (data not shown).

Quantitative histologic analysis. *Epidermal thickness.* Analysis of the epidermal thickness of limbs in each of the laser-irradiated groups indicated that the epidermal thickness of limbs irradiated for five days increased significantly ($P < 0.05$) compared with limbs irradiated for three days. The epidermal thickness of the sham control irradiated group increased from 36.2 μ ± 1.1 on day 3 to 75.5 μ ± 4.1 on day 5 of culture. A similar increase in epidermal thickness was observed in all the laser-irradiated groups after both three and five days of laser irradiation.

Collagen / noncollagenous protein ratios. The collagen/noncollagenous protein ratios obtained from the dermis after three days of laser irradiation were significantly ($P < 0.05$) increased above sham controls in limbs irradiated with lower energy densities of laser (0.23, 1.37, and 2.75 J/cm^2; Fig. 4A). By day 5 in culture, dermal collagen/noncollagenous protein ratios increased further ($P < 0.05$) in the sham controls and in the limbs receiving laser irradiation at an energy density of 1.37 and 2.75 J/cm^2 (Fig. 4A). Significant differences were not found on day 5 in dermal collagen/noncollagenous protein ratios between sham controls and any of the laser-irradiated limbs. The collagen/noncollagenous protein ratio

determined in the bone region of sham control limbs increased progressively between days 3 and 5 of culture (Fig. 4B). A similar increase in bone collagen/noncollagenous protein ratios between days 3 and 5 of culture was observed in limbs irradiated with laser, except those irradiated with 2.75 and 3.66 J/cm^2.

A low-energy laser, by definition, is one that produces a temperature elevation of no greater than 1.0°C [1]. Because the limbs used in the present study did not contain intact circulatory systems, the ability to dissipate heat may have been compromised, resulting in temperature elevations, which could constitute a thermal effect. To exclude such an effect, the temperature of the tissue culture media surrounding the limb was monitored during laser irradiation at each energy density by using an indwelling temperature probe. Laser irradiation at any energy density did not produce a detectable increase in the tissue culture media.

DISCUSSION

The present results demonstrate for the first time that laser irradiation at an energy density as low as 0.23 J/cm^2 can affect the growth and development of fetal mouse limbs maintained in an organ culture system. The observed effects occurred in isolated fetal mouse limbs in the absence of intact nerves or blood vessels and therefore were likely due to a direct action of laser irradiation on the fetal tissues. Statistically significant differences were found in both morphological and histologic appearances of limbs irradiated with lower energy densities of laser over a 3-day period in culture. The direct effects of low-energy densities of laser irradiation produced a significant reduction in the amount of new tissue growth and marked changes in the histologic appearance and a significant increase in the amount of collagen deposited in the dermis of fetal limb sections. Despite these dramatic changes observed following three days of irradiation with low levels of laser, higher energy densities of laser irradiation did not have a significant effect on the growth and histologic characteristics of fetal mouse limbs. Furthermore, no differences were detected in limbs irradiated with any energy density of laser for the five-day period in culture.

Examination of tissues by a blinded observer showed significantly increased histologic scores for dermal cell number and for thickness and amount of collagen fibers in limbs irradiated with the lower energy densities of laser over the three-day period in culture. These histologic observations are substantiated by the results obtained from computer image analysis demonstrating increased collagen deposition in the dermis of limbs irradiated with the lower energy densities of laser. Based on dramatic changes in collagen deposition in both the quantitative and qualitative histologic evaluations of the dermis of fetal limbs, it is hypothesized that laser irradiation resulted in a stimulation of the proliferation and secretion of dermal fibroblasts, which is consistent with results from previous studies performed both in vitro and in vivo. Increased cellular proliferation after laser irradiation of cultured human oral and rat skin fibroblasts has been documented [22,23]. Laser-irradiation-induced increased collagen production [24–26] has also been documented in in vitro cell culture studies. Studies of healing in animals have demonstrated enhanced collagen accumulation in the wound area, with resultant elevated tensile strength of the wounds as a result of laser irradiation [27–32]. Alterations in the transcription of mRNA of procollagen type I has been reported as a mechanism by which laser irradiation stimulates collagen production in fibroblasts [33,34]. Another possible mechanism is an increase in mitochondrial transmembrane electrical potential caused by absorption of light energy resulting in the stimulation of the synthesis of adenosintriphosphate (ATP) [35,36], resulting in an increase in the synthetic activity of fibroblasts [24]. Laser irradiation has also been shown to transform fibroblasts into myofibroblasts, modified actin-rich fibroblasts involved in granulation tissue contraction [5,37]. Studies have indicated that laser irradiation can induce the production of basic fibroblast growth factor [38] and the release of growth factors from macrophages [39,40]. Whereas most studies have suggested that laser irradiation can alter fibroblast activity, some studies have shown no effect on DNA synthesis activity [26] or on fibroblast proliferation [41–43]. The absence of significant increases in the dermal collagen/noncollagenous protein ratios after irradiation of limbs at higher energy densities (2.75, 3.66, and 4.58 J/cm^2) is in agreement with results of previous studies that have also shown that laser causes inhibition of collagen production and fibroblast proliferation at higher energy densities [25,41–43]. Lower energy densities of irradiation result in Ca^{2+} transport into the cytoplasm, which in turn triggers mitosis and enhances cell proliferation [44]. At higher energy densities of laser

irradiation, too much Ca^{2+} is released, causing hyperactivity of Ca^{2+}-ATPase calcium pumps, which exhausts the ATP reserves of the cells [44].

We report in the present study that all limbs demonstrated a significant increase in the thickness of the epidermis over the 5-day period in culture. No significant differences were found in the amount of thickening of the epidermis in limbs irradiated with laser compared with limbs in the control group. Previous studies, performed on cultured human keratinocytes, have demonstrated that laser irradiation increases certain cellular activities of epidermal cells [45,46]. It has been suggested that there is no phototherapeutic response in cases in which cell proliferation is active and regeneration is occurring at a maximal rate [35]. Minimal or no effect was present after laser irradiation of fresh wounds [35], whereas a great effect has been shown on cultures grown in poor conditions [47]. This observation may explain why biostimulation is not always possible and may account for the lack of significant differences in epidermal thickness of limbs irradiated with laser compared with sham controls in the present study.

All irradiated groups exhibited increases in the bone collagen/noncollagenous protein ratios over the 5-day period in culture. However, no significant differences were found in the collagen/noncollagenous protein ratios measured in bones of limbs irradiated with laser compared with the sham controls. In vivo studies have indicated that laser irradiation may have a beneficial effect on wound healing of bone by accelerating bone regeneration [48], stimulating formation of trabecular osteoid tissue [49], increasing vascularization [50], and promoting faster metabolism and reaction of bone callus by modulation of the function of osteocytes [50]. An increase in callus stiffness and the maximum load at failure and enhancement in bone repair have been reported in the fractured tibia of rats irradiated with laser [51,52]. Alterations in alkaline phosphatase and tartrate-resistant acid phosphatase activity suggest that laser irradiation could affect the population of osteoblasts and osteoclasts in the injured site [52]. The absence of an effect on collagen/noncollagenous protein ratios and histologic scores of bone in the present study suggests that the effect of laser irradiation on bone may be observed in in vivo studies, mediated through circulatory and neural factors, and be absent in a serum-free organ culture system.

Macroscopic examination of the limbs after three days of laser irradiation showed that limbs irradiated with lower energy densities of laser had significantly lower amounts of new tissue growth and no change in limb length compared with limbs in the sham control group. Histologic examination of these same limbs showed significant augmentation of the number of cells and collagen fibers present in the dermis and in the quantity of dermal collagen/noncollagenous protein ratios assessed. Therefore, continued limb growth and new tissue formation was arrested in limbs that were undergoing rapid cell differentiation. The absence of a change in gross limb appearance despite the dramatic changes observed at the cellular level suggest that measures of fetal outcome based on morphometry, such as fetal size and weight, may not provide an accurate indication of the effects of an exogenously applied stimulus on fetal growth and development.

Although the limb development occurring in the in vitro organ culture system used in the present study is not equivalent to the in vivo uterine environment, all limbs demonstrated an increase in new tissue growth, dermal collagen deposition, and epidermal thickness over the five-day period in culture. In addition, the changes observed in the histologic characteristics of limb tissue are consistent with limb development that occurs normally in vivo, indicating that the continual growth and differentiation of the fetal limbs occurs in the organ culture system. It is suggested that many of the effects of laser irradiation are mediated through cell constituents that are released into the general circulation. For example, untreated wounds on the contralateral sides of animals irradiated with laser as opposed to untreated wounds of untreated control animals have shown an increase in the tensile strength [30]. Therefore, the use of a serum-free organ culture system facilitates the study of the direct effects of laser irradiation on fetal tissue by eliminating circulatory and neural factors while preserving individual cells and the associated extracellular matrix. This experimental model permits the delivery of a known energy density of laser directly to the fetal limbs without passage through intervening maternal tissues. Furthermore, by using this experimental model, it is also possible to control factors that alter laser dosimetry such as the distance to the target tissues and the angle of incidence of the laser beam.

The results of the present study indicate that laser irradiation directly affects the growth and development of day 14 fetal limbs in an in vitro

system at an energy density as low as 0.23 J/cm^2. To account for issues pertaining to the depth of laser penetration and systemic effects, future studies are required that examine the effects of laser irradiation on fetal growth and development in an in vivo model. Measures of fetal outcome based on morphometry may not provide an accurate indication of the effects of an exogenously applied stimulus on fetal growth and development. Changes that occur at the cellular level during intrauterine development may not exhibit themselves as changes in gross fetal appearance. Until more information is available on the effects of laser irradiation on the fetus in utero, the use of low-level laser in obstetrics should be avoided. The present result does not preclude the use of laser for wound healing and pain modulation for musculoskeletal conditions in other patients.

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